

APPLICATION
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TITLE: ARTIFICIAL LIVER APPARATUS AND METHOD

APPLICANT: EDWARD F. MYERS, ALBERT P. LI, ACHILLES DEMETRIOU

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Edward Myers
THE 2nd APPL

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Artificial Liver Apparatus and Method

This application is a continuation-in-part of U.S.
5 Patent Application Serial No. 07/943,777, filed September
11, 1992, now abandoned.

BACKGROUND OF THE INVENTION**1. Field of the Invention**

10 This invention relates to an device and a process for
the extracorporeal purification of blood and plasma.
Specifically, it relates to a purification device which,
while similar in structure to hemodialysis devices used
in the treatment of renal insufficiencies, uses novel
15 biological means to perform many of the functions of a
normal human liver. The device and method are therefore
intended to assist in the treatment and support of
patients suffering from liver disease or who have
undergone transplantation of liver tissue.

2. History of the Prior Art

20 To best understand the invention, an overview of
principles of anatomy and physiology relating to human
liver function and disease is useful. The liver is an
organ divided into two principal lobes made up of
functional units called lobules. A lobule consists of
25 cords of hepatic cells arranged radially around a central
vein. Between these cords are sinusoid spaces lined with
phagocytic cells known as Kupffer cells. Oxygenated
blood is provided to the liver via the hepatic artery
while deoxygenated blood leaves the liver via the hepatic
30 portal vein. Branches of these vessels deliver blood to
the sinusoids, where oxygen, most nutrients and certain
toxins are extracted into the blood.

35 Through the liver, excess glucose is removed and stored
as the polysaccharide glycogen. When the level of glucose
in the blood drops below normal, glycogen will be broken
down into glucose which is released into the blood stream.

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into the blood stream. The liver also assists protein metabolism by extracting and storing excess amino acids in the bloodstream for use in the construction of many plasma proteins, such as albumin. Bile, a solution of salts, bilirubin, cholesterol and fatty acids which assists in the emulsification of fats and intestinal absorption of lipids, is also produced by hepatic cells. It is not, however, normally secreted into the bloodstream by these cells but is instead transported to, and stored in, the gallbladder.

Of greater importance to this invention is not the liver's role in digestion of food but its role in regulating the concentration of wastes and toxins in the blood. Hepatic cells contain enzymes which either break down toxins carried in the blood, transform them into less harmful substances or, failing either of those processes, stores them. For example, metabolism of amino acids will result in the release of free amino acids and nitrogenous wastes, the latter of which are converted by hepatic cells to urea. In moderate amounts, this urea is harmless and is easily excreted by the kidneys and sweat glands. "Old" red blood cells and certain bacteria can also be destroyed and, in the case of the former, recycled by the Kupffer cells.

In short, the liver is vital to maintaining the body's normal biochemical state. Impairment or loss of its function can, therefore, be fatal. A concise summary of known possible derangements of hepatic metabolism can be found in Podolsky, et al., "Derangements of Hepatic Metabolism" Ch. 315, *Principles of Internal Medicine*, 10th ed., pp. 1773-1779, 1983. The medical art has developed

humane as well as many other species can regenerate regenerating lost or damaged liver tissue.

However, although supportive and pharmaceutical treatments to transplant patients have been developed

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many symptoms of liver disease, these methods all require time which an acutely ill patient may not have. Further, while undergoing treatment, support for any loss of normal liver function must be provided to maintain or approximate 5 metabolic homeostasis. A means, therefore, is needed which can perform the cleansing functions of the liver when it cannot, thus increasing the time available for treatment.

Extracorporeal liver perfusion (i.e., pumping blood 10 through foreign liver tissue) has been a proposed means for treatment and support for many years, with mixed success. An example of the use of repeated liver perfusions for long-term hepatic support can be found in Abouna, et al., "Long-Term Hepatic Support by Intermittent 15 Liver Perfusions", *The Lancet*, pp. 391-396, (Aug. 22, 1970), which reports maintenance of a patient suffering from liver failure for 76 days using periodic liver perfusions. However, despite attempts to use liver tissue from 5 different species, immunological and other 20 biochemical reactions limited the use of the perfusions and the patient died before a suitable transplant donor could be found.

Isolated hepatocyte transplantation has also been performed, again with mixed results (see, e.g., Makaowa, 25 L., et al., *Can. J. Surg.*, 24:39-44, 1981, and Demetriou, et al., *Proc. Natl. Acad. Sci. USA*, 83:7475-7479, 1986).

In contrast, extracorporeal methods of purifying blood and plasma; i.e., by hemodialysis, hemoperfusion or hemofiltration are well-known and established in the art 30 for treatment of renal insufficiencies. The major goal of these methods is to maintain fluid and electrolyte balance in the patient.

35 containing an artificial semipermeable membrane suspended in a dialysis solution. With a concentration gradient established across the membrane for a particular solute that is to be removed, the solute will diffuse across the

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occur. This method can be used to successfully lower the concentration in blood of urea and in plasma of potassium. Net removal of substances whose concentrations should not be altered in blood or plasma, such as sodium in the
5 latter, can be removed by establishing a hydrostatic pressure gradient across the membrane, creating a convective pathway for movement of solutes across the membrane. Details concerning the structure of a conventional hemodialysis device as well as means for controlling fluid temperature, dialyzate concentration, and fluid flow therein are set forth in several existing patents, including, representatively, U.S. Patent No.
10 5,011,607 to Shinzato, U.S. Patent 4,923,598 to Schal, U.S. Patent 4,894,164 to Polaschegg, and U.S. Patent 5,091,094 to Veech.
15

In operation, blood is removed or pumped directly from the patient into the dialyzer and flows along one side of the membrane. The dialysis solution is pumped in counterflow across the membrane; effluent blood is returned to the patient.
20

Hemodialysis according to the method outlined above is most effective for the removal of small molecular weight species that are water-soluble and not protein-bound. As a result, it is principally used in therapy for renal insufficiencies, although it may be used in the
25 treatment of certain drug overdoses. To use the method effectively to compensate for loss of liver function, however, additional strategies for blood detoxification are required.

To that end, a number of implantable and extracorporeal bioartificial liver devices have been proposed in the art and tested in animal models.
30

Although the design and operation of these devices have varied widely, they share common elements. For example, the devices typically utilize isolated
35 hepatocytes or hepatocyte equivalents to provide metabolic

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through as well as one or more permeable membranes. Of the devices which utilize isolated hepatocytes, the hepatocytes are typically anchored onto a supporting substrate to facilitate cellular differentiation and aggregation.

For example, using many of the concepts disclosed in the parent application of this continuation-in-part application, a bioartificial liver containing hepatocytes bound to collagen-coated microcarrier beads (CYTODEX 3 beads, a trademarked product of Pharmacia) in a hollow fiber containing bioreactor was tested and described as producing more efficient metabolite transfer than systems which entrap hepatocytes within gel or gel droplets (Rozga, et al., *Biotech. and Bioengineering*, 43:645-653, 1994; see also, Miura, et al., *Artif.Org.*, 10:460-465, 1986 [re use of a calcium alginate gel as a cell support], and Cai, et al., *Artif.Org.*, 12:383-393, 1988 [microencapsulation of cells in a gel]).

Other approaches to the use of isolated hepatocytes in an artificial liver have attached the cells to microcarriers and placed them into a chromatography column for perfusion (Demetriou, et al., *Ann.Surg.*, 259-271, 1986), onto hollow fibers (Jauregui, et al., *J.Cell Biochem.*, 45:359-365, 1991; see also, U.S. Patent No. 5,043,260), onto glass plates stacked in a module and perfused with oxygenated medium (Uchino, et al., *ASAIO Proc.*, 34:972-977, 1988), onto asialoglycoprotein polymers (Akaike, et al., *Gastroenterol.*, 28 Supp. 45-52, 1993), and into beds packed with matrix-forming materials such as glass beads (Li, et al., *In Vitro Cell Dev.Bio.*, 29A:249-254, 1993). The efficacy of these approaches has

35 formed cell aggregates, immobilized nutrient transfer between the cells and nutrients, metabolites and toxins (as a result of immobilization of the cells onto a substrate which limits a portion of the cell's ability to

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Over time, techniques to improve cell viability and aggregation have been improved (see, e.g., published patent application 93-272876 [WO 9316171], which describes a system similar to that disclosed in Li, et al., *In Vitro Cell Dev.Bio.*, *supra*). However, it has been generally accepted in the art that hepatocyte aggregation and function sufficient for use in extracorporeal liver support are dependent at least on attachment of the cells to a substrate or matrix, if not also immobilization of the cells (see, e.g., Rotem, et al., *Biotech. and Bioengineering*, 43:654-660, 1994 [hepatocytes are anchorage dependent cells]; Miura, et al., *Biomatter Artif.Cells Artif.Org.*, 18:549-554, 1990 [hepatocyte aggregation is required for proper cell function; to that end, immobilization of the cells is preferred]; Rozga, et al., *Ann.Surg.*, 217:502-511, 1993 [attachment of cells to microcarriers enhances cell function and differentiation]; and, Rozga, et al., *Biotech. and Bioengineering*, *supra* [attachment of cells to microcarriers or entrapment of cells in a gel preferred]). In contrast, conventional hemodialysis utilizing a membrane against a suspension of free (i.e., "unattached"), isolated hepatocytes has not been shown to be clinically effective in providing liver support (see, e.g., Olumide, et al., *Surgery*, 82:599-606, 1977). Thus, the bioartificial liver devices that utilize isolated hepatocytes which are presently being developed and tested in the art anchor the cells to a substrate, a process which entails a relatively delicate manufacturing step informing the cell/substrate attachment, and risks damage to the cells.

SUMMARY OF THE INVENTION

35 A method of reducing cellular intoxication in a biological system such as blood or plasma, whereby blood or separated plasma is circulated through a bioreactor having at least one semi-permeable membrane allowing interpenetration of the

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semipermeable membranes may be in tube, film or hollow fiber form (preferably the latter), and are surrounded by a sterile cell culture medium in solution for maintenance of hepatocytes and/or a hepatoma cell line (as explained further below, the term "hepatocyte" will, unless context otherwise requires, refer both to isolated hepatic cells and a combination of those cells with Kupffer bile duct epithelial and endothelial cells and, in some instances, fibroblasts). Soluble proteins, glucose and toxins in the blood or plasma diffuse across the membrane into the culture medium for metabolism by the hepatocytes.

In one embodiment, the hepatocytes utilized in the invention are attached to biologically compatible microcarrier particles. Circulation of the microcarriers in the cell culture medium is assisted by means such as an alternating magnetic field to affect magnets embedded within the microcarriers. As the diffused molecules (i.e., solutes) come into contact with the bound hepatocytes they are taken up by the cells and broken down, transformed or stored according to normal hepatic cell function with respect to said molecules. Effluent blood or plasma (after recombination with a previously separated fraction of red blood cells) is then returned to the patient.

In the preferred embodiment of the invention, unattached hepatocytes (i.e., cells which are not attached to a substrate or otherwise immobilized) are utilized. Means are provided to facilitate cell aggregation within the device (i.e., *in situ*) to provide extracorporeal liver support to a patient in need of such support.

Additional purification means such as a hemofiltration dialysis means for removing waste products from the culture medium may also be provided as needed to remove certain toxic drug or waste products not broken down or stored by the hepatocytes (such as urea excreted thereby into the culture medium). Means may also be provided to

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the device to remove any antibodies formed to the hepatocytes or, for example, to xenogeneic grafts of liver tissue in transplant patients not captured by the hepatocytes.

5 **BRIEF DESCRIPTION OF THE DRAWINGS**

FIGURE 1 is a schematic representation of a preferred embodiment of the device of FIGURE 1 for use with unattached hepatocytes.

10 FIGURE 2 is a schematic representation of an alternative embodiment of the device of the invention, including preferred additional purification means.

15 FIGURE 3 is a graph illustrating the percentage of rat hepatocytes viable after up to 21 hours of circulation of a population of hepatocytes through the device of FIGURE 1. Viability was determined by trypan blue exclusion.

FIGURE 4 is a graph illustrating the percentage of porcine hepatocytes viable after up to 6 hours of circulation of a population of hepatocytes through the device of FIGURE 1.

20 FIGURE 5 is a graph illustrating the percentage of rat hepatocytes viable after up to 6 hours of circulation of a population of hepatocytes through the device of FIGURE 1 as used for *in vivo*, extracorporeal purification of plasma of a male pig.

25 FIGURE 6 is a graph illustrating the extent to which the ammonia concentration within fluid circulating through the blood loop of the device of FIGURE 1 decreased over time with circulation of a population of rat hepatocytes through the hepatocyte loop of FIGURE 1.

30 FIGURE 7 is a graph illustrating the extent to which the urea concentration within fluid circulating through the blood loop of the device of FIGURE 1 decreased over time with circulation of a population of rat hepatocytes through the hepatocyte loop of FIGURE 1.

35 **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

A. Isolation and Preparation of Cells.

A principal feature of the invention is the ability

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living, isolated liver cells to assist in the purification of substances out of blood or plasma which would, absent liver disease, impairment or failure, be removed by the patient's own liver. For most embodiments, the cells will 5 be hepatocytes of nonhuman, human, or xenogeneic origin. However, it will be understood by those of skill in the art that hepatoma cells may also be used as the liver cells of the invention. Thus, while hepatocytes are the preferred liver cells due to the lesser risk of pathogenesis that they pose, the word "hepatocyte" and the disclosure herein will be understood to encompass hepatoma cells, such as the human hepatoma cell line Accession No. 10 CRL 8024, which is available from the American Type Culture Collection, Rockville, Maryland.

15 Preparation of hepatocytes for use in the invention is as follows:

Methods for isolation of human hepatocytes suitable for use in the method of the invention are known in the art (see, e.g., Takahashi, et al., *Artif.Org.*, 17:653-659, 20 1993). The use of human hepatocytes in an artificial liver system may reduce the risk of immunologic reaction by the patient to contact with nonhuman hepatocytes. However, the availability of human hepatocytes is necessarily limited. Therefore, of nonhuman hepatocytes, 25 porcine hepatocytes are preferred for use in the invention principally because of their availability and physiologic similarity to human hepatocytes. It is also anticipated that, for use of the system with liver transplant patients, porcine hepatocytes will be particularly useful 30 where porcine tissue can be modified to be a xenogeneic organ source for human transplantation. It will be appreciated that other modifications can be made to the invention without departing from the spirit and scope thereof.

35 The hepatocytes of the invention:

Using porcine tissue as an example, therefore, hepatocytes in lobules are retrieved from the liver immediately following slaughter of the animal. The

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lobular tissue is screened for disease according to means known in the art. Hepatocytes are then isolated from the lobular tissue by enzymatic (collagenase) digestion according to means known in the art and purified (see,
5 e.g., techniques described in Berry, et al., J. Cell.
Biol. 43:506-520, 1969; Seglen, Methods Cell Biol. 13:29-
83, 1976; and Example I, *infra*). Depending on the method
of isolation used, Kupffer cells and other cellular
10 components of the lobular tissue (such as bile duct
epithelial cells) may also be included with the
hepatocytes. If desired, the hepatocytes may be further
purified by pelleting the cells at low speed (e.g., 50
Xg), in a reagent such as the 60% PERCOLL solution
supplied commercially by Sigma Chemical of St. Louis, MO.

15 Because the biochemical states which contribute to or
cause liver disease are not completely understood, it is
not yet known the extent to which each of these cell
populations may contribute or be necessary to the function
of the invention. It is expected that the use of purified
20 populations of hepatocytes will be preferred; however, as
used herein, the term "hepatocyte" should be understood
to encompass hepatic cells or fibroblasts alone or in
combination with Kupffer cells, bile duct epithelial and
endothelial cells. It may also be desirable to utilize
25 cells which have been genetically altered to produce or
metabolize liver specific products such as albumin or
clotting factors. These cells could be used exclusively
or in a cocktail with native hepatocytes.

Once purified, one or more hepatocytes may be attached
30 for use in one embodiment of the invention to a
biologically inert carrier, preferably beads of 150-200

Under the trade name HYDÉP™, Biomedicine AB
35 Biotechnology of Uppsala, Sweden. Equivalent materials
such as agarose beads (available from Bio-Rad, Inc. of
Richmond, California) or other suitable supports can be used.

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may also be used. Therefore, although for convenience the substrate to be used to support the hepatocytes in the invention will be referred to as beads, those of skill in the art will know of, or can readily ascertain, other support substrates suitable for use in this embodiment of the invention.

The beads will preferably be coated with denatured collagen or equivalent biological material to permit attachment of the hepatocytes thereto. Collagen is the preferred surface because exogenous fibronectin is not required for attachment of hepatocytes to collagen.

Examples of suitable methods can be found in "Microcarrier Cell Culture: Principles and Methods" available on request from Pharmacia LKB Biotechnology, in Gjessing, et al., *Exp. Cell. Res.* 129:239-249, 1980, and in Demetriou, et al., *Science*, 23:1190-1192, 1986, the disclosures of which are incorporated herein by this reference to illustrate knowledge in the art concerning cell attachment to microcarrier substrates. For example, using a commercially available spinner vessel, 10 mg. of dextran beads can be added to each milliliter of cell culture. Hepatocytes, to a final concentration in the cell culture medium of 1×10^5 cells/ml., are added to the beads, mixed gently and incubated at 37°C. Periodic stirring may be necessary as the cell culture medium volume is increased if attachment is not achieved readily.

However, in the preferred embodiment of the invention, the hepatocytes will not be attached to a substrate or otherwise immobilized. Therefore, for use in this embodiment of the invention, hepatocytes will be isolated as described above and will be stored as described below.

The attached hepatocytes will be harvested and washed with a medium containing 10% fetal calf serum and 10% heat-inactivated horse serum. The hepatocytes will then be resuspended in a medium containing 10% fetal calf serum and 10% heat-inactivated horse serum and will be frozen and stored in a container of liquid nitrogen and maintained according to substantially the same methods used to preserve immortalized hepatocytes.

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artificial insemination. A particularly suitable method for cryopreservation of hepatocytes is described in Rozga, et al. *Biotech. and Bioengineering*, 43:645-653, 1994. An alternative method for cryopreserving hepatocytes is 5 described in Dixit, et al., *Transplantation*, 55:616-662, 1994, wherein cells are encapsulated and frozen in calcium alginate. By freezing the hepatocytes, it may be possible to store the cells for periods of several years, although storage for periods of less than a year would be 10 preferred. However, most preferably, to the extent practical, the hepatocytes will be used shortly after isolation.

For use of the hepatocytes, the cells will be suspended in a biocompatible culture medium. If frozen, 15 the cells may be thawed and washed in a suitable buffer, such as phosphate buffered saline. Preferably, the culture medium will contain nutrients and other supplements to maintain the viability of the hepatocytes, which nutrients and supplements are known to, or may be 20 readily ascertained by, those of ordinary skill in the art. An example of a culture medium suitable for use in hepatocyte maintenance is the medium sold under the trade name RPMI-1640 by Grand Island Biologicals, Grand Island, New York. Another suitable and commonly used culture 25 medium is the WAYMUTH medium supplied commercially by GIBCO Laboratories, Grand Island, NY. Preferably, the medium will be supplemented with salts or nutrients known to those skilled in the art depending on the needs of the particular cells used in the system (i.e., which may vary 30 depending on whether cells other than hepatocytes are included, the length of use of the hepatocytes, the specific substances to be metabolized).

35 common substances such as alanine, arginine, asparagine, albumin, aminoleulinic acid, oleic acid, dexamethasone, thyroxine, tocopherol, glucagon, insulin and gentamicin, all of which are commercially available and suitable

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supplemented culture medium is described in detail in Example I, *infra*. Alternatively, particularly if the hepatocytes are to be used for purification purposes within about 6 hours of isolation or thawing, the culture 5 medium may be any blood compatible fluid, such as saline.

B. Bioartificial Liver Devices Suitable for Use in the Methods of the Invention

1. Construction and Operation of a Device Preferred for Use With Unattached Hepatocytes

10 Referring now to the drawings, FIGURE 1 depicts a preferred embodiment of the device of the invention for use primarily with unattached hepatocytes, while FIGURE 2 depicts suitable modifications of the device of FIGURE 1. The devices of the invention are generally composed of a purification bioreactor having at least one semipermeable membrane therethrough, and at least two pumps or other means for circulation of the hepatocytes and blood or plasma through the device. Generally, each 15 device encompassed by the invention will include a first conduit (i.e., biological fluid loop 20) and a second conduit (i.e., hepatocyte loop 5), which conduits are separated by at least one semi-permeable membrane. All of the components of the device (including connectors, clamps, luer-locks, tubing, and containers) will 20 preferably be sterilizable.

In addition, means for sensing and controlling flow temperature and pressure of the dialyzate and blood, as well as determining the relative concentrations of solutes therein, are known in the art and, where noted below, may 25 be suitable for use in the device of this invention. For purposes of illustration, reference may be made for these

30 substantially at body temperature and should be returned to the patient at substantially the same temperature. Maintenance of this third temperature will be accomplished

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external temperature control means for blood and plasma known to those skilled in the art; e.g., means used to maintain the temperature of fluids during plasmapheresis, blood bank storage or transfusion. Means for temperature control of fluid during renal hemodialysis and/or hemofiltration are also described in U.S. Patent 4,894,164 (disclosing a technique for cooling blood exposed to heated dialyzate during renal hemodialysis). A proposal for maintaining the blood temperature at 34°C could also be utilized with the process of this invention (see, e.g., Maggiore, et al. Proc. EDTA 18:597-602, 1981). An oxygen source (i.e., oxygenator 9) will also be provided to supply oxygen to the hepatocytes.

One particularly suitable means for temperature control places most of the components of the device within an insulated incubator sufficient to maintain the temperature of the fluids circulating within the device at about body temperature (e.g., 37°C for humans). Suitable incubator materials will be known to, or can be readily ascertained by those of skill in the art and include foam or glass in a durable container, such as stainless steel.

It should be noted that operation of the entire system, including control of the valve means and the initiation and cessation of flow into and out of mixing vessel 1 and stations within the hepatocyte loop 5, as well as flow into, through and out of the biological fluid loop 20, will preferably be substantially continuous within a purification cycle. Control of the system will preferably be by control means (not shown) such as a microprocessor with manual override capabilities according to means well known in the art.

Alternatively, or preferably, in addition to these electronic control means, manual control means (not shown) will be provided throughout the system to allow a skilled operator to open and close the various valves and pumps as

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of each station within biological fluid loop 20, control the pump means 8, 14 and 15, and control flow into and out of hepatocyte loop 5.

In addition, although pump means are depicted in FIGURE 1 at particular locations, it will be appreciated by those skilled in the art of mechanical engineering that one or more of such pump means may be included in the loops at sites other than the ones shown.

Referring to FIGURE 1, a preferred embodiment of the device includes a mixing vessel 1 into which unattached and uncontaminated (i.e., "new") hepatocytes will be introduced and circulated to facilitate their aggregation prior to introduction of the cells into the purification bioreactor 7 via hepatocyte loop 5. The mixing vessel 1 will include a hepatocyte introduction port 2 (for introduction of new hepatocytes into mixing vessel 1), sampling port 3 (for the removal of contaminated (i.e., "old"), hepatocytes and culture medium from mixing vessel 1), hepatocyte circulation outlet port 4, hepatocyte circulation inlet port 5 and a waste outlet 6. Conveniently, these ports may be formed of tubing attached to and through a wall of mixing vessel 1.

For example, where mixing vessel 1 is formed of glass, ports 2-6 may be formed of glass tubes fused into and through the wall of vessel 1 at suitable locations, such as those shown in FIGURE 1. The ports may also be attached to mixing vessel 1 with a luer-lock or equivalent fitting attached to the port via flexible tubing that can be closed using a hose clamp or similar clamping means. Other designs and materials for ports 2-6 will be apparent to those of ordinary skill in the art and will not, therefore, be described in detail.

Identical and/or similar ports may be formed in mixing vessel 1 using flexible tubing or biologically compatible materials similar or identical to those used to form mixing vessel 1. It is anticipated that such ports will be located in the same relative positions as the ports shown in FIGURE 1.

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be sealably attached to mixing vessel 1 at outlet port 4 and inlet port 5. Conveniently, the seal attachment may be made by an O-ring and O-ring joint clamp, the latter of which will allow adjustments to be made to increase or 5 decrease the tension on the O-ring seal.

The mixing vessel 1 and purification bioreactor 7 must be of a biologically compatible, non-cytotoxic material and will be preferably be of a polymer compound such as acrylonitrile butadiene styrene ("ABS") resin plastic, 10 polypropylene, polysulfone, glass or equivalent materials composed to meet USP XXI Class VI toxicity testing standards.

The size of mixing vessel 1 may vary, but will generally be sufficient to retain a number of hepatocytes 15 equivalent to at least 1% of the approximate weight of the liver in the mammalian species to be treated; i.e., a "theoretical minimum" number of cells. For example, in humans, the theoretical minimum number of cells which would be expected to be effective in extracorporeal 20 purification of blood or plasma is about 1% of the liver's hepatocytes or about 2 billion cells. The mixing vessel will, therefore, be sufficient in size to contain at least a theoretical minimum number of hepatocytes suspended in a sterile, biologically compatible fluid such that the 25 hepatocytes may be circulated within mixing vessel 1; i.e., the cells will not be packed to the point of immobilization within the mixing vessel.

The size of the purification bioreactor 7 will depend 30 on the volume of blood or plasma to be circulated through the system. It is expected that this volume will vary from 1/2 pint to 4 pints, with the former being the volume expected to be circulated in a pediatric application.

35 The purification bioreactor 7 may be used as a plasma expander in plasma blood products.

Purification bioreactor 7 is in fluid communication 40 with a source of biocultural filters and a dialysis membrane.

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to be treated by external connections which form a circulation loop for the biological fluid (e.g., blood or plasma) to be treated. The purification bioreactor will preferably be a hollow fiber bioreactor. In a hollow
5 fiber bioreactor, the circulation of biological fluid through purification bioreactor 7 will occur through a multiplicity of capillaries (representatively depicted as 7' in FIGURE 1) formed of semipermeable membranes which will allow the exchange therethrough of soluble proteins
10 (which may include antibodies) and metabolic waste products of sizes which will vary according to the application.

To this end, the membrane pore size is expected to vary upward to a pore large enough to permit passage of
15 proteins therethrough, with the larger pore size intended for removal of proteins such as antibodies. It should be noted that where larger pore sizes are used, certain desirable substances (e.g., plasma proteins) may diffuse into the culture medium. Means for return of these
20 substances are provided below; because of this phenomenon, however, it is expected that pore sizes of about 0.2 μm in diameter will be most practical and will result in significant fluid convection, which is the primary force responsible for solute transport across the membranes that
25 form the hollow fibers.

A commercially available and suitable product for use as purification bioreactor 7 is the ZYMAX hollow fiber bioreactor (ZYMAX is a registered trademark of Microgon, Inc. of Laguna Hills, California). The ZYMAX product comprises a collection of 3600 hollow fibers formed of a semipermeable membrane material having a pore size of 0.2 μm formed of mixed ester poly-

meretheran. The fibers are contained within a ported, 35 biologically compatible and non-cytotoxic polysulfone housing; fittings, including clamps, gaskets, hoses and plenum components are supplied with the system.

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manufacturer. Other suitable hollow fiber bioreactors are the Z22M-060-01X bioreactor from Microgon (which has only 670 fibers, but uses fibers of longer length [about 550 mm potted end] than many conventional bioreactors), and
5 [OTHER EXAMPLES?????????]

The total number of hollow fibers (shown as 7' in FIGURE 1) required is dependent on the volume of culture medium contained within purification bioreactor 7, which is in turn dependent on the volume of biological fluid to be purified within the inventive system (which will determine the number of hepatocytes to be suspended within the culture medium). For example, given a known volume of blood or plasma and the volume and permeability of the hollow fibers (approximately 200 milliliters in the lumens
10 and 0.2 μm respectively for a single ZYMAX bioreactor), the total number of fibers and volume of culture medium can be estimated. As a general principle, the total surface area of the hollow fibers should be maximized with respect to the volume of culture medium to ensure
15 contacting of solutes from the blood and plasma with hepatocytes circulating through purification bioreactor 7 in hepatocyte loop 5.

Within purification bioreactor 7, hepatocyte loop 5 comprises the extracapillary space within the bioreactor
25 housing, while biological fluid loop 20 comprises the intracapillary space; i.e., the lumens of the hollow fibers 7' contained within bioreactor 7. The movement of hepatocytes through the hepatocyte loop is driven by pump 14 (e.g., a peristaltic pump), while the movement of blood
30 or plasma through the biological fluid loop is driven by pump 8 (e.g., a blood monitor pump).

As indicated in the background section,

35 hepatocytes are aggregated in the culture aggregate together. For example, it has been previously shown that unaggregated, single hepatocytes in culture die relatively rapidly compared to cells in aggregates. For example,

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prior art artificial liver systems provide an extracellular solid substrate to allow the hepatocytes to aggregate within a bioreactor and/or culture the cells in a manner which encourages the formation of aggregate spheroids before use of the cells in an artificial liver system.

The process by which hepatocytes aggregate requires energy. Thus, although not absolutely necessary to their survival, the viability of hepatocytes for use in an artificial liver system has been shown to be enhanced by the presence of oxygen, particularly in the early stages of aggregation (see, e.g., Rotem, et al., *Biotech. & Bioengineering*, 43:654-660, 1994 [oxygen partial pressure of about 0.064 mmHg required for half-maximal attachment of a single layer of hepatocytes to a collagen-based substrate, while about 0.13 mmHg was shown to be required for the cells to spread across the substrate]). Where hepatocytes in an artificial liver system or culture are present in multiple layers, it has been suggested that oxygenation of the cells beneath the surface of the top layer may require shortening the diffusion distance between hepatocytes and the oxygen source or increasing the concentration of oxygen present in the early stages of aggregation (Rotem, et al., *supra* at 659).

In the preferred embodiment of the invention, the hepatocytes are neither immobilized onto a solid substrate nor precultured into aggregate spheroids. Instead, the cells are introduced directly into a circulation suspension of blood compatible fluid (preferably saline or a hepatocyte culture medium as described *supra*) and encouraged to aggregate *in situ*. This utilization of the hepatocytes of the invention will result in a

35 aggregation of the cells in a manner which allows each cell the entire surface of each cell is available for interaction with other cells, oxygen, nutrients and metabolic waste products without the need for a solid

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damage to each cell inherent in the handling involved in preculturing the or immobilizing the cells, and shortens the time between isolation and use of cells in the method of the invention.

5 To ensure adequate oxygenation of unattached hepatocytes in the circulation suspension, mixing vessel 1 is filled with a blood compatible fluid which is driven by pump 15 from a feed reservoir 13 or other fluid source through line 18. The fluid is oxygenated by oxygenator 10 9, which may be any conventional fluid oxygenator known in the art. For oxygenation, recycle pump 14 is activated such that the fluid in line 18 passes into line 19 through a valved feed line 12 at outlet port 4 and through oxygenator 9. Leaving oxygenator 9, the culture medium 15 passes pH probe 10 and dissolved oxygen probe 11.

The pH and oxygen probes will be set at desired set points and the gas composition of the culture medium adjusted accordingly. The culture medium will be maintained at a pH of about 7-8, preferably about 7-7.5, 20 and most preferably about 7.35. The oxygen composition of the culture medium will be maintained between about 0 and 20%, preferably not lower than about 5%. Most preferably, the oxygen composition will be about that of saturated air at the time that the hepatocytes are 25 introduced into the culture medium. The oxygen composition is adjusted by increasing or decreasing the concentration of oxygen introduced into the culture medium in oxygenator 9, while the pH of the culture medium may be controlled by increasing or decreasing the 30 concentration of carbon dioxide introduced into the culture medium at oxygenator 9, as appropriate. The balance of the gas composition is controlled by the

35 addition of additional gas components to the circulation fluid. As the culture medium enters mixing vessel 1 through outlet port 4, gas leaves the system through waste outlet port 5. The waste outlet port 5

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Flow through the biological fluid loop will be as follows. Blood or plasma for purification will be either removed from the patient for introduction into the system or, preferably, flow will be generated directly from the patient. In the preferred embodiment, vascular access will be provided according to medically accepted techniques such as those used for acute or chronic renal dialysis (e.g. catheterization, direct anastomosis of native vessels or artificial grafts).

In the most preferred embodiment, the biological fluid for purification will be plasma. After removal of blood from the patient, the plasma fraction is separated from the red blood cells by plasmapheresis, using a method and device (not shown) well known in the art. Plasmapheresis may be performed independently and out of the biological fluid loop, but the means therefor will preferably be included in the loop so that direct vascular access to the patient is with the plasmapheresis device.

The plasma fraction (or unseparated blood where plasmapheresis is not performed) passes through biological fluid loop 20 to hollow fibers 7' via a connection conduit (not shown) to each fiber or, for the preferred embodiment using a bioreactive housing such as the ZYMAX product, to each such housing wherein said conduits will be provided and attached thereto according to the housing manufacturer's specifications. The structure of the conduits may vary widely according to the number and structure of the hollow fibers 7' used, but will be formed of a biologically compatible, non-cytotoxic material similar to that disclosed above for use to form mixing vessel 1 or an arterial flexible tubing known in the art.

After passing through the means for purification (such as, for example, pressure regulator 24 shown in FIGURE 2) so effluent blood returned to the patient is at an adequate pressure.

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and are not, therefore, described further herein.

Flow of blood or plasma through biological fluid loop 20 can be generated by the pressure gradient between the fluid pressure of the blood or plasma as it leaves the patient, but will preferably be assisted by a low pressure mechanical pump means 8 preceding the input portal to biological fluid loop 20, which will preferably be fitted with a pressure regulator (not shown) to prevent any back surge. A suggested placement of flow sensor 25 and control means 30 are schematically depicted in FIGURE 2. As suitable means for flow control are well-known in the art, no further description thereof will be provided here. It may also be necessary to provide means for release of air (not shown) from purification bioreactor 7; again, such means are well-known in the art and not described further here.

Noncellular components of blood or plasma introduced into bioreactor 7 via biological fluid loop 20 will exchange across the membranes comprising hollow fibers 7' with the culture medium in the hepatocyte loop. Once the exchange is complete, hepatocytes are introduced into mixing vessel 1 through hepatocyte introduction port 2, then additional culture medium added as needed to ensure that the mixing vessel 1 remains completely filled with fluid. Alternatively, the hepatocytes may be introduced into mixing vessel 1 as the culture medium is being exchanged with the plasma.

In this respect, those of skill in the art will appreciate that the use of unattached hepatocytes requires a delicate balance to be achieved between the flow rate in the hepatocyte loop sufficient to facilitate contact between, and aggregation of, the hepatocytes.

As will be appreciated, the quantity of blood necessary to detoxify a therapeutically beneficial quantity of blood or plasma must be balanced against the need to optimize the rate at which an unattached hepatocyte population can

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which comprise hollow fibers 7' into the hepatocyte loop (i.e., into the extracapillary space of bioreactor 7) will occur.

To this end, the direction of flow in the biological fluid loop 20 will be substantially opposite the direction of flow in the hepatocyte loop 5. Through activation of recycle pump 14 in a direction opposite that used to oxygenate the culture medium, the cell-containing circulation fluid in the hepatocyte loop will flow from mixing vessel 1 through bioreactor 7, past probes 10 and 11 and through oxygenator 9 at a rate of about 20 to 80 milliliters of fluid/minute, preferably at a rate of about 65 milliliters of fluid/minute. At the same time, through activation of blood monitor pump 8, blood or plasma in the biological fluid loop will be maintained at a rate of about 20 to 250 milliliters fluid/minute, with a rate of 150-200 milliliters fluid/minute being preferred.

It should be noted that the flow rate in the biological fluid loop which may be effectively and simply controlled through the use of a fluid reservoir. By retaining blood or plasma in this reservoir, a store of fluid is available for processing according to the method of this invention at a substantially higher flow rate and volume than would be possible if flow was directly and entirely from the patient. Using the reservoir, therefore, fluid would be collected from the patient, directed to the reservoir, then removed through valve means and pumped through the biological fluid loop as otherwise described herein.

Returning to the passage of flow through biological fluid loop 20, as blood or plasma passes through hollow fibers 7', soluble proteins and metabolites enter the lumen of the hollow fibers and the culture medium in

bioreactor 7, as well as the so-called transmembrane pressure.

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It will be appreciated that, unlike the process of renal dialysis, no counterflow across hollow fibers 7' is normally provided or desired in this device. Although it can be expected that there may be some back diffusion of 5 solutes which reach a concentration equilibrium vis-a-vis the fluid in loop 20 and the culture medium, such diffusion is preferably avoided by replenishment of the culture medium, compensated by additional purification means (e.g., those shown as 40 and 50 in FIGURE 2), as 10 well as minimized by the concentration gradient across hollow fibers 7'.

Counterflow may be important, however, where the pore size of the membrane is required to be large; e.g. when 15 diffusion of larger molecular weight species out of blood is sought. With those species, however, may for example diffuse plasma proteins which should be returned to the patient. In such an application, a multiplicity of channels may be used to capture plasma proteins and return them to the biological fluid loop utilizing a counterflow 20 (see, e.g., the counterflow pump means described in U.S. patent No. 5,011,607). For example, if the filtrate to be removed were known to be a certain diameter, smaller plasma proteins (e.g., albumin) could be captured by a second and smaller semipermeable membrane bounded 25 oppositely by a channel (not shown) leading back to biological fluid loop 20. The remaining filtrate, being too large to diffuse across the second membrane, would remain in solution in the culture medium for metabolism by the hepatocytes.

30 Alternatively, in absence of means to return desirable solutes to the patient, such solutes, in particular plasma proteins, may be independently returned to the patient...

Foreign blood products.

As a general principle, proteins and waste products (hereafter "solutes") in addition to the culture media

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susceptible to being metabolized or stored by the hepatocytes will be absorbed by those hepatocytes which they contact. However, it is expected that the degree of absorption achieved will be limited in at least two respects.

First, certain solutes will remain in solution because they do not contact a hepatocyte, or do not contact a viable hepatocyte; i.e., one capable of absorption of filtrate. It is this latter possibility that gives rise to the second limitation; i.e., hepatocytes may die, become damaged or otherwise be rendered incapable of performing their expected functions in the system.

As it would not be possible to determine or predict when function may be lost to a given hepatocyte, it may be necessary to replace all or a percentage of the hepatocytes used in the invention through sampling port 3. However, as used in the device and according to the method described above, the hepatocytes circulating in the device should remain viable for at least about 6 hours, the period of time which would be expected to be expended in a single purification treatment of an adult human.

Further, because solutes not absorbed by hepatocytes (due to the factors discussed above or, as would be true of certain toxins, because the solutes are not susceptible to being metabolized by the liver) will remain in the culture medium, additional removal means may be required and periodic replacement of the culture medium will be necessary.

To that end, hepatocyte loop 5 may include a replenishment station (not shown) at sampling port 3 wherein flow through the loop will be temporarily interrupted for removal from and replacement

variety of structures, including a sterile and ported tank or conduits having valve means (not shown) from which "used" cells and medium would flow and into which new

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cells and medium would be introduced.

Preferably, this removal and reintroduction would occur at predetermined intervals throughout a purification cycle to ensure optimal performance of the process.

5 Because it would be somewhat impractical to recycle all of the hepatocytes and culture medium during a single purification cycle, removal and replacement of a statistical percentage thereof is preferred. This percentage, as well as the intervals at which it will be removed and replaced, will vary according to the volume of medium, cells and biological fluid in the system and, once these values are known, can be determined according to accepted statistical calculations known in the art.

10 Alternatively, or in conjunction with the above method, 15 the entire volume of cells and medium could be replaced after each cycle.

On completion of a purification cycle, blood is returned to the patient or plasma returned first to the plasmapheresis device for recombination with the patient's red blood cells, then returned to the patient via conduits identical to those described above for introduction of blood or plasma into biological fluid loop 20. It will be appreciated that an immunoassay for IgM and/or known liver function tests may be performed to assist in verification of proper and effective performance of the inventive process prior to or after return of the blood to the patient. Depending on the course of treatment indicated and length of cycle required, the purification cycle could be repeated up to two or three times in a single day.

With respect to the additional removal means, once flow in biological fluid loop 20 has passed through

35 the additional removal means, the biological fluid loop 20 is returned to the patient or, alternatively, before being passed again through bioreactor 7. Alternatively, and depending on the substance to be additionally removed,

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these means could be included within hepatocyte loop 5. For example, it will be appreciated that for certain metabolic waste products excreted into the culture medium by the hepatocytes; e.g., urea, removal means will be best situated in the hepatocyte loop 5.

As the biochemical states which contribute to or cause liver failure or damage may vary according to clinical cause (e.g., hepatitis, alcohol or toxic contamination, drug overdose) and are not completely known or understood, it is not clear what extracorporeal liver functions will be required for a particular patient. Nor is it clear which of those functions will be completely or competently performed by the hepatocytes, and which may have to be completed or performed by additional means. However, examples of such means are well known in the medical arts, are representively depicted in FIGURE 2 as additional purification means 40 and 50, and may include:

- Adsorbent means for removal of solutes such as urea onto an activated charcoal column or, coated charcoal (such as the filter sold under the trade name "GAMBRO") other resin adsorbents (a commercially available adsorber is available as the "DT" machine [detoxifier] from Ash Medical, Inc. West Lafayette, Indiana).

- Conventional dialysis means for removal of small molecular weight species that are water soluble and not strongly bound to protein(s).

- Immunoreactive procedures to remove natural IgM antibodies present in human serum that can react with porcine xenoantigens. Examples of immunoabsorbent materials include dialysis membranes sold by VWR Scientific under the trademarks SPECTRA/POR 6, SPECTRA/POR 7 and SPECTRA/POR 1. Lectins attached to cellulose

isolation and purification means may also be used manually or by automation according to techniques well known in the art; an example of such means is the commercial product

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Pierce, Rockford, Illinois, under its trademark IMMUNOPURE (IgM Purification Kit).

5 - Means for hemofiltration (i.e., means where blood is passed through an extracorporeal circuit through a hemofilter by which an ultrafiltrate of uremic toxins is withdrawn and an equal quantity of uremic-toxin-free blood is added in proportion the volume of blood via an exchange element).

10 The presence or absence of such additional purification means in either loop will increase or decrease the time needed to complete a given purification cycle by the time needed to perform each additional purification step. It will be appreciated, however, that these steps may be performed through use of the means 15 described independent of the inventive system.

It should be understood that sound medical practice dictates that bioreactor 7 may, and preferably will, be disposed of and replaced or sterilized following each purification cycle and will not be used with more than one patient. It is expected that, depending on the course of treatment indicated, each purification cycle would last several hours if performed at a constant rate. In addition, it will be appreciated by those of ordinary skill in the art that more than one bioreactor may be utilized in parallel by providing additional conduit, fitting and pump means such as those described *supra* with respect to the single bioreactor system depicted in FIGURES 1 and 2.

30 **C. Construction and Operation of a Device For Use With Attached Hepatocytes in the Method of the Invention.**

It will be appreciated by those of skill in the art that the device described *supra* may be used to aggregate hepatocytes.

10 It is not necessary to use a flow rate which is necessary to insure that the flow rate of fluid in the hepatocyte loop is sufficient to facilitate cell aggregation. Instead, the rate of flow will be controlled so as to

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between the attached hepatocytes is minimized to avoid damage thereto. In addition, the attached hepatocytes may be introduced directly into bioreactor 7 via a replenishment station and/or sampling port in the 5 hepatocyte loop. To this end, the device may be simplified as schematically depicted in FIGURE 2.

The use of attached hepatocytes in an artificial liver system presents the drawbacks discussed *supra*, such as decreased surface area for contact between the hepatocytes 10 and solutes to be metabolized. To compensate for the loss of cellular surface area, means are provided in this embodiment of the device to circulate the hepatocytes within the bioreactor.

This circulation must, however, be performed gently 15 to avoid contacting the inner surface of bioreactor 7, thus damaging or destroying the hepatocytes. Because of this concern, mechanical mixing of the culture medium or shaking of bioreactor 7 are possible but not preferred methods of achieving circulation of the hepatocytes within 20 the bioreactor. Instead, a preferred method of circulation is the application of an alternating magnetic field to the bioreactor.

According to this method, a low frequency AC power supply 31 (e.g., 60 cycles/second) and at least one pair 25 of opposing coils 32 electrically connected thereto are used to generate an alternating, low frequency magnetic field. Coils 32 will preferably be situated opposite one another and externally to bioreactor 7; preferably, they will be housed in a nonconductive housing and attached to 30 opposite external walls of the reactor by any suitable attachment means. A very moderate circulating flow within the culture medium is generated.

35 The microcarriers 23A to which the hepatocytes are attached (shown in FIGURE 2 as beads 23A having an "X" therethrough representative of the magnetic particles within anisotropic binding material) are magnetically suscep-

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will be placed in at least 1 out of approximately 10 beads). Beads having magnetic particles therein are commercially available from Dynal, Inc. of Great Neck, New York and are sold under the trade name DYNABEADS M-450.

5 Another useful method of achieving circulation while maximizing contact of solutes with the attached hepatocytes is achieved using a boundary layer pump. Most useful with film or sheet (rather than hollow fiber) membranes, a boundary layer creates a stream of culture
10 medium which moves along the face of the membrane and carries attached hepatocytes therewith. Alternatively, and particularly if the use of hollow fiber membranes is desired, hepatocytes and culture medium could be introduced into an axial conduit (not shown) through the
15 inner diameter of fibers of a sufficient diameter to accommodate said conduit and passed therethrough using a pump to generate centripetal force to spiral or circulate the hepatocytes and medium in the conduit.

Boundary layer pump means, and the use thereof to move
20 solid articles, are described in U.S. Patent No. 4,335,994 to Gurth. Such pump means (not shown) are available from Discflo Corporation in Santee, California and are sold under the trademark DISCFLO PUMP. Instructions for and assistance with the attachment ~~and~~ use of the DISCFLO PUMP
25 are available from Discflo Corporation.

Except for these modifications, the operation and structure of this alternative embodiment of the device of the invention is as disclosed with respect to the preferred embodiment described *supra*.

30 Examples regarding the use of the inventive devices and methods are provided below. The examples are intended to illustrate rather than limit the invention.

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EXAMPLE I

IN VITRO EVALUATION OF THE ARTIFICIAL LIVER

DEVICE OF FIGURE 1 USING UNATTACHED HEPATOCYTES

Hepatocytes were isolated from Sprague-Hawley rats
5 and/or farmer's pigs after slaughter using the technique
described in Li, et al., [CITE?], 1990. Briefly, the
technique was performed using collagenase (0.5% w/v, type
1) perfusion. The isolated cell population was determined
to have over 80% viability as assessed by trypan blue
10 exclusion. Following isolation, the hepatocytes were
suspended in WAYMUTH 752/L culture medium supplemented
with 11.2 mg/l alanine, 12.8 mg/l serine, 24 mg/l
asparagine, 0.168 mg/ml aminolevulinic acid, 0.393 mg/l
dexamethasone, 0.03 mg/l glucagon, 20 units/ml insulin, and
15 84 mg/l gentamicin.

The mixing vessel, bioreactor and biological fluid
loop were filled with culture medium and equilibrated to
a 20% oxygen concentration in a total medium volume of
about 350 ml. Ammonia in a concentration of about 20
20 ug/ml was added to the biological fluid loop as a model
waste product for metabolism in the artificial liver
device. Approximately 200 million viable rat hepatocytes
were injected into the mixing vessel, while an equivalent
number of porcine hepatocytes were injected into the
25 mixing vessel of a second device. Circulation of fluid
through the hepatocyte and biological fluid loops was
begun and maintained at a flow rate of about 65 ml per min
in both loops.

The number of rat hepatocytes still viable in the
30 system after 21 hours is graphically depicted in FIGURE
3, while the number of porcine hepatocytes still viable
after 6 hours (the total test duration)

35 was determined by conventional trypan blue
i.e., trypan blue exclusion staining.

FIGURES 5 and 6 respectively depict the decrease in
ammonia concentration obtained in the culture media

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both devices over a period of 6 hours. As shown in the FIGURES, a substantial decrease in ammonia concentration was obtained. It is predictable based on these data and the data reported in Example II, *infra*, that the ammonia concentration in blood or plasma can be reduced in a human by a factor of about 10%.

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EXAMPLE II

IN VIVO EVALUATION OF THE ARTIFICIAL LIVER

DEVICE OF FIGURE 1 USING UNATTACHED HEPATOCYTES

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The device of FIGURE 1 was prepared for use with rat hepatocytes as described in Example I, except that culture medium was not introduced directly into the biological fluid loop. A venous connection between the biological fluid loop of the device and the femoral veins of a young male pig (weight=about 26 kg) was established. Blood flowing from the femoral veins of the pig was perfused through the bioreactor for about 6 hours at a flow rate of about 150 ml/min, with continuous return of blood to the pig.

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The vital signs and blood chemistry of the pig were observed throughout the 6 hour perfusion period. Hepatocytes were collected from the mixing vessel through the sampling port periodically and the viability of the cells evaluated by trypan blue exclusion staining.

FIGURE 5 depicts the percentage of hepatocytes still viable during the perfusion period at the time points indicated in the FIGURE.

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FIGURE 6 depicts the decrease in ammonia concentration measured in blood sampled from the biological fluid loop of the device at the time points indicated in the FIGURE. FIGURE 7 depicts the increase in urea concentration.

AS shown in the FIGURES, the observed efficacy of the system and viability of the hepatocytes circulated through the system as used in vivo was comparable to that observed

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efficacy and viability measurements reported in Example II. The pig did not suffer any detrimental health effects attributable to the use of the artificial liver device.

Although preferred embodiments of the inventive device and methods are disclosed herein, it will be appreciated by those skilled in the art that modifications may be made to the embodiments disclosed without departing from the spirit or scope of the invention, which is defined by the claims appended hereto.